Purification and characterization of N-acetyl-β-D-glucosaminidase from the Italian honey bee, *Apis mellifera ligustica* (Hymenoptera: Apidae)

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Abstract: 【Aim】 N-acetyl-β-D-glucosaminidase (NAGase) is an important chitinase for degradation of chitin. It can cleave terminal N-acetyl-β-D-glucosamine residues from the nonreducing ends of N-acetyl-β-D-glucosides, participating in the ecdysis of exoskeletons in insects. Researching and characterizing this enzyme from honey bees may help to clarify its mechanism of action in the development of honey bees. 【Methods】 NAGase was purified from larvae of the Italian honey bee (*Apis mellifera ligustica*) by means of $(NH_4)_2SO_4$ fractionation (40% – 70% of saturation), DEAE-cellulose ion exchange chromatography, and Sephadex G-100 gel filtration. The enzyme activity was determined by using p-nitrophenyl-N-acetyl-β-D-glucosaminide (pNP-NAG) as the substrate. The purity of the enzyme was determined by native PAGE and SDS-PAGE. The isoelectric point (pI) of the enzyme was measured by IEF-PAGE, and its molecular weight was determined by Sephadex G-200 gel filtration. 【Results】 The specific activity of purified NAGase was 803.09 U/mg and the molecular weight was 77.3 kD. The enzyme was composed of two subunits with the same molecular weight of 39 kD. Its pI was determined to be 4.8. Results of kinetic analysis indicated that the enzyme in the hydrolysis of pNP-NAG followed Michaelis-Menten kinetics with the Michaelis-Menten constant (K_m) of 0.11 mmol/L and the maximum velocity (V_m) of 17.65 μmol/L · min, respectively. The optimum pH and optimum temperature of the enzyme for hydrolysis of pNP-NAG was pH 5.5 and 60℃, respectively. And the activation energy of the enzyme for the hydrolysis of pNP-NAG was determined to be 64.8 kJ/mol. Pb^{2+}, Cu^{2+}, Zn^{2+} and Al^{3+} inhibited the enzyme activity in different degrees. 【Conclusion】 In this study the enzyme NAGase has been purified and characterized from *A. mellifera ligustica*, laying a foundation for further unveiling the structure and function of NAGase in bees.

Key words: *Apis mellifera ligustica*; N-Acetyl-β-D-glucosaminidase; purification; characterization; kinetics

1 INTRODUCTION

Chitin, one of the most abundant organic compounds found in nature, is widely distributed as a principle component of insect exoskeletons, shells of crustaceans and fungal cell walls (Fajardo-Somera *et al.*, 2015; Younes *et al.*, 2016; Kappel *et al.*, 2016). In insects, chitin is found in the cuticle of the integument and the peritrophic membrane of the midgut (Kelkenberg *et al.*, 2015; Alvarenga *et al.*, 2016). Insects must undergo periodic shedding or molting of the cuticle for their growth and maturation (Zen *et al.*, 1996). Chitinase plays an important role in degradation of chitin, especially the cuticle formation. Endo-chitinases can cleave chitin randomly.

N-acetyl-β-D-glucosaminidase (NAGase, EC 3.2.1.52), which hydrolyzes N-acetylated chitooligosaccharides to GlcNac, can cleave terminal N-acetyl-β-D-glucosamine residues from the nonreducing ends of N-acetyl-β-D-glucosides (Nagamatsu *et al.*, 1995). Hence, numerous researches have been done on this enzyme from many species. Microbial chitinolytic enzymes have been investigated extensively for their potential use in the enzymatic production of GlcNac and its oligomers in an eco-friendly manner (Huang *et al.*, 2012). In human, NAGase exists in various organs, especially in kidney, so it is now used as a biomarker to diagnose kidney injury (Doi *et al.*, 2012; Katagiri *et*

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al., 2012). In insects, NAGase was researched extensively as a potential biological target for biopesticide (Liu *et al.*, 2009). NAGase has been isolated and characterized from various insect species, such as *Bombyx mori* (Nagamatsu *et al.*, 1995), *Ostrinia furnacalis* (Liu *et al.*, 2009) and *Pieris rapae* (Shi *et al.*, 2007).

Honey bees are holometabolous insects with a life cycle of four stages including egg, larva, pupa and adult. During the larval and pupal stages, bees experience the process of molting and the growth of the peritrophic membrane. NAGase, an important enzyme for the hydrolysis of chitin, massively exists in larva. Hence, we purified and characterized this enzyme from the Italian honey bee, *Apis mellifera ligustica*. The basic research of NAGase can contribute to clarifying its mechanism of action during the development of honey bees.

2 MATERIALS AND METHODS

2.1 Test insects

A. m. ligustica larvae were obtained from the Bee Science College of Fujian Agriculture and Forestry University and treated in accordance with the guidelines regarding animal welfare established by the People's Republic of China. All procedures were pre-approved by the Institutional Animal Care and Use Committee of Fujian Agricultural and Forestry University.

2.2 Reagents

DEAE-cellulose (DEAE-32) was purchased from Whatman. Sephadex G-100 and G-200 were Pharmacia products. Protein marker was Amersham product. p-Nitrophenyl-N-acetyl-β-D-glucosaminide (pNP-NAG) was purchased from the Biochemistry Lab of Shanghai Medicine Industry Academy (Shanghai, China). All other reagents were local products of analytical grade. The water used was re-distillated and ion-free.

2.3 Purification of the enzyme

One hundred g larvae of *A. m. ligustica* were homogenized in 250 mL 50 mmol/L Tris-HCl buffer (pH 7.5) containing 0.2 mol/L NaCl and incubated for 2 h. Then the homogenate was centrifuged at 3 000 g for 30 min. The precipitate was removed and the supernatant was collected as the crude enzyme. The crude NAGase was fractionated with $(NH_4)_2SO_4$. The 40% – 70% saturated $(NH_4)_2SO_4$ fraction was collected by centrifugation (10 000 g for 30 min), dissolved in a small amount of 50 mmol/L Tris-HCl buffer (pH 7.5), and then dialyzed against this buffer until no sulfate could be detected. All operations above were done at 4℃.

The crude enzyme was concentrated by ultrafiltration, and then put on DEAE-cellulose (DEAE-32) column (2.6 cm × 30 cm) to equilibrate with 300 mL 50 mmol/L Tris-HCl buffer (pH 7.5). Elution was carried out with a linear gradient of 0 – 1.5 mol/L NaCl at a flow rate of 20 mL/h in 300 mL 50 mmol/L Tris-HCl buffer (pH 7.5), and 3 mL per tube was collected. Fractions with enzyme activity were pooled. The active fractions were combined and applied to further chromatography on Sephadex G-100 gel filtration column (2.6 cm × 60 cm), the column was washed with equilibrium buffer of 300 mL 50 mmol/L Tris-HCl buffer (pH 7.5, containing 0.2 mol/L NaCl). Elution was carried out with a flow rate of 20 mL/h in 300 mL 50 mmol/L Tris-HCl buffer (pH 7.5) and 3 mL per tube was collected. Fractions with enzyme activity were pooled. Pooled fractions were all dialyzed against 50 mmol/L Tris-HCl buffer (pH 7.5) and then stored at –20℃ for further research. All purified processes were performed in a chromatographic ice cuber at the temperature of 4℃. The final preparation was applied to native polyacrylamide gel electrophoresis (PAGE) for the assay of purity. The concentration of separation gel was 7.5%.

2.4 Determination of enzyme activity and protein concentration

Enzyme activity was determined at 37℃ by following the increasing absorbance at 405 nm accompanying the hydrolysis of the pNP-NAG (Xie and Chen, 2004). A portion of 10 μL of enzyme solutions was added to the reaction media (2.0 mL) containing 0.5 mmol/L pNP-NAG in 0.15 mol/L NaAc-HAc buffer (pH 5.5). After reaction for 10 min at 37℃, 2 mL of 0.5 mol/L NaOH was added into the reaction mixture to stop the reaction. Absorption was recorded using a Beckman UV-650 spectrophotometer. The molar absorption coefficient of the product pNP is 1.73×10^4 mol/L · cm (Prody *et al.*, 1985; Katagiri *et al.*, 2012). One unit of enzymatic activity was defined as the amount of enzyme catalyzing the formation of 1 μmol/L pNP-NAG at the conditions above denoted. The protein concentration was measured according to the method described by Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard.

2.5 Determination of molecular weight of enzyme subunit

SDS-PAGE was done on a discontinuous horizontal thick-layer with a stacking gel 3% acrylamide in Tris-HCl buffer (pH 6.7) at 10 mA current and a separating gel of 12% acrylaimide in

Tris-HCl buffer (pH 8.9), 0.1% SDS at 20 mA current. Both NAGase sample and marker were combined with 1-fold solubilizing mixture containing 1% SDS, 1% 2-mercaptoethanol, 40% sucrose, 0.02% bromophenol blue (tracking dye) and 0.01 mol/L Tris-HCl buffer (pH 8.0). The mixtures were heated in a boiling water bath for 3 min. After electrophoresis, the gel was stained with 0.05% Coomassie Brilliant Blue R-250. The electrophoretic migration distance of the standard proteins was converted to the electrophoretic relative mobility (m_R) by using the equation: $m_R = L_s/L_b$; where L_s is the electrophoretic migration distance of the sample, L_b is the electrophoretic migration distance of the bromophenol blue. A logarithmic curve was obtained by plotting the electrophoretic relative mobility (m_R) versus the molecular mass.

2.6 Determination of the isoelectric point (pI) of enzyme

Isoelectric focusing PAGE (IEF-PAGE) (electrophoresis tube: 5 mm × 100 mm) was also done to determine the pI of this enzyme. The pH range of ampholyte was 3.0 – 9.5.

2.7 Determination of the molecular weight of enzyme

The molecular weight was determined using gel filtration on Sephadex G-200 and SDS-PAGE. The gel filtration was done on Sephadex G-200 (2.5 cm × 70 cm) with 0.01 mol/L Tris-HCl buffer (pH 7.5, containing 0.2 mol/L NaCl). Pepsin (PEP, 35.0 kD), ovalbumin (OA, 45.0 kD), bovine serum albamin (BSA, 68.0 kD), rabbit phosphorylase B (RP, 97.2 kD) and bovine gamma globulin (BGG, 140.0 kD) were used as the standard proteins. A calibration curve was obtained by plotting the average elution volume of samples of three replications (V_e) versus the logarithm of the molecular mass. The native molecular mass of the enzyme was calculated from the calibration curve.

2.8 Determination of the kinetic parameters of enzyme

The kinetic behavior of the enzyme in the hydrolysis of pNP-NAG was studied. The enzyme activities for the hydrolysis of pNP-NAG at various concentrations (0.05 – 2 mmol/L) were tested at pH 5.5 and 37℃. Lineweaver-Burk plot was used to determine the kinetic parameters of Michaelis constant (K_m) and maximal velocity (V_m) values.

2.9 Assay of the optimal pH and pH stability of enzyme

The optimum pH of the enzyme was determined by measuring the enzyme activities as described in section 2.4 at different pH values at 37℃. The pH stability of the enzyme was monitored by incubating the enzyme in different pH buffers including Gly-HCl (pH 2.5 – 4.0), NaAc-HAc (pH 4.0 – 5.8), Na_2HPO_4-NaH_2PO_4 (pH 5.8 – 8.0) and Gly-NaOH (pH 8.0 – 10.6) for 20 h at 4℃, respectively. Sixty μL of the mixture of enzyme and buffer was used for activity assay in the standard system (37℃, pH 5.5).

2.10 Assay of the optimal temperature and thermal stability of enzyme

The optimal temperature of the enzyme was determined by measuring the activity as described in section 2.4 at various temperatures (10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85 and 90℃) at pH 5.5. The thermal stability of the enzyme was monitored by incubating the enzyme in different temperatures (10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85 and 90℃) for 30 min. Then a portion of 60 μL of the mixture was taken for activity assay as described above.

2.11 Determination of the activation energy of enzyme

The activation energy of the enzyme was determined by measuring the reaction velocity at different temperatures (30, 37, 40, 45 and 50℃). At each temperature, the maximum velocity (V_m) was assayed and then the Arrhenius formula was used to calculate the activation energy by making a plot of $\lg V_m$ versus $1/T$ (T was absolute temperature). The slope of the line was the activation energy of enzyme to catalyze the hydrolysis of pNP-NAG.

2.12 Effect of metal ions and EDTA on enzyme activity

The activity of the purified enzyme was measured in the standard system (37℃, pH 5.5) described in section 2.4 with different concentrations (5, 10, 15, 20, 30, 40 and 50 mmol/L) of various metal ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+}, Al^{3+} and Pb^{2+}) and EDTA, and expressed as the relative activity percentage calculated from the ratio of the specific activity of NAGase treated with metal ions or EDTA to that of untreated NAGase.

3 RESULTS

3.1 Purification of NAGase and determination of its molecular weight and subunit number

The crude enzyme of NAGase was concentrated and loaded in DEAE-32 anion-exchange column. A single activity peak was obtained (Fig. 1: A). The active fractions were pooled and applied to chromatograph by Sephadex G-100 gel filtration column (Fig. 1: B). The activity peak was overlapped with

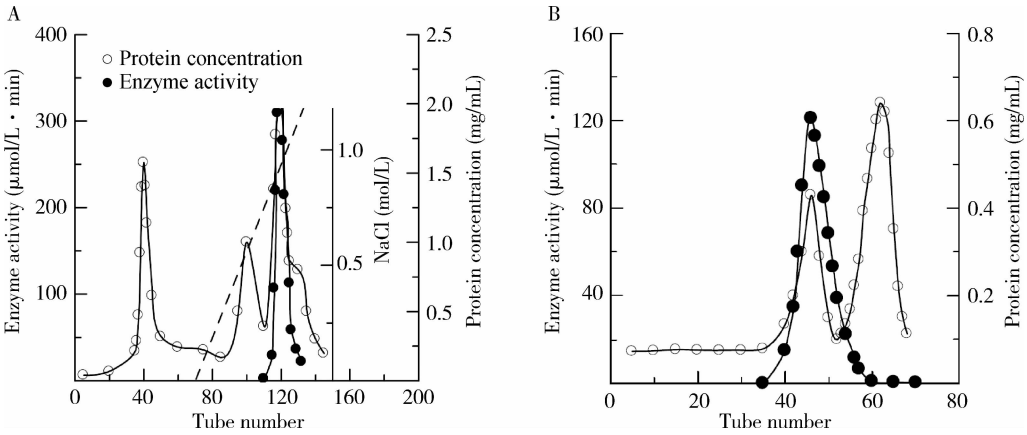

Fig. 1 Purification of NAGase from *Apis mellifera ligustica* larvae
A: DEAE-cellulose (DEAE-32) column chromatography of NAGase; B: Sephadex G-100 column chromatography of NAGase.

the protein peak and the active fractions were pooled. The final enzyme showed a single band on native PAGE (Fig. 2: A) and SDS-PAGE (Fig. 2: B). The specific activity of the purified NAGase was determined to be 803.09 U/mg. All the purification steps were summarized in Table 1.

The molecular weight of each enzyme subunit was estimated to be 39.0 kD by SDS-PAGE (Fig. 2: B). On the other hand, the enzyme molecular weight was determined by gel filtration on Sephadex G-200 column, the result was showed in Fig. 3. The molecular weight of the enzyme was estimated to be 77.3 kD. These results indicated that the enzyme was composed of two subunits with the same molecular mass. The pI of the purified NAGase was determined to be 4.8 by IEF-PAGE (data not shown).

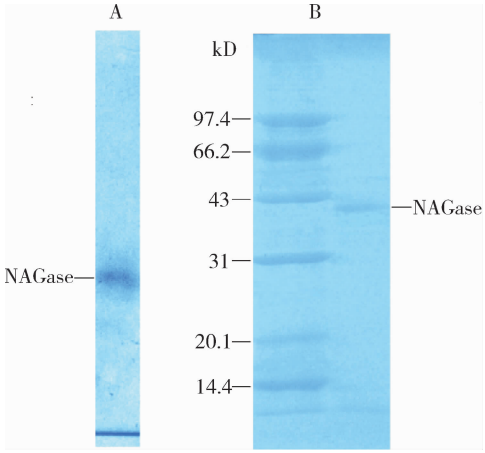

Fig. 2 Native PAGE (A) and SDS-PAGE (B) of the purified NAGase from *Apis mellifera ligustica* larvae

Table 1 Purification of NAGase from *Apis mellifera ligustica*

Steps	Total activity (U)	Total protein content (mg)	Specific activity (U/mg)	Purification (fold)
Crude extract	122 727.29	4 139.10	29.65	1.00
$(NH_4)_2SO_4$ fractionation	59 120.45	773.76	76.41	2.58
DEAE-32 ion exchange chromatography	23 834.32	129.27	184.38	6.22
Sephadex G-100 gel filtration	9 516.62	11.85	803.09	27.09

3.2 Determination of the kinetic parameters of NAGase

The kinetic behavior of NAGase in the hydrolysis of pNP-NAG was studied under the conditions of 0.15 mol/L NaAc-HAc buffer (pH 5.5) and different concentrations of pNP-NAG at 37℃. The enzyme concentration was 0.2 mg/mL. Under this condition, the hydrolysis of pNP-NAG by NAGase followed Michaelis-Menten kinetics (Fig. 4). The kinetic parameters of NAGase obtained from a Lineweaver-Burk plot showed that the K_m and V_m values were 0.11 mmol/L and 17.65 μmol/L · min, respectively.

3.3 Optimum pH, pH stability, optimum temperature and thermal stability of NAGase

The optimum pH of NAGase for hydrolysis of pNP-NAG was determined. The results showed that the optimum pH of catalytic reaction was pH 5.5. The pH stability of the enzyme was also determined. The results indicated that 90% of enzyme activity was exhibited between pH 4 – 8, and the enzyme would be fairly unstable out of this pH range (Fig. 5: A).

The enzyme activities were measured at various

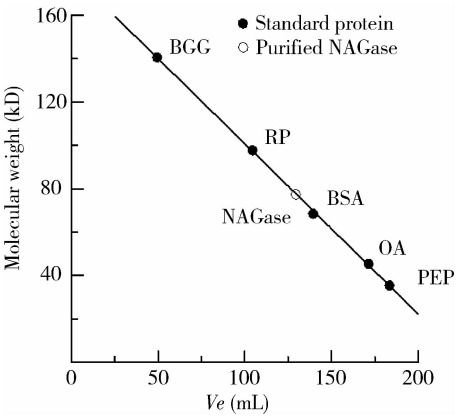

Fig. 3　Measurement of the molecular weight of NAGase on Sephadex G-200 gel filtration

BGG: Bovine gamma globulin; RP: Rabbit phosphorylase B; BSA: Bovine serum albamin; OA: Ovalbumin; PEP: Pepsin.

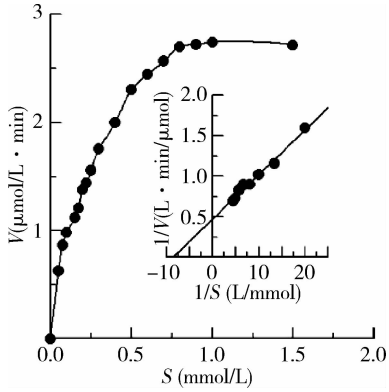

Fig. 4　Michaelis-Menten kinetics for the hydrolysis of pNP-NAG by NAGase

temperatures (10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85 and 90℃) to discuss the effect of temperatures on the enzyme activity. The results showed that the optimum temperature of catalytic reaction of NAGase was 60℃. After incubation under different temperatures for 30 min, the enzyme was stable below 65℃ and rapidly inactivated above 65℃ (Fig. 5: B).

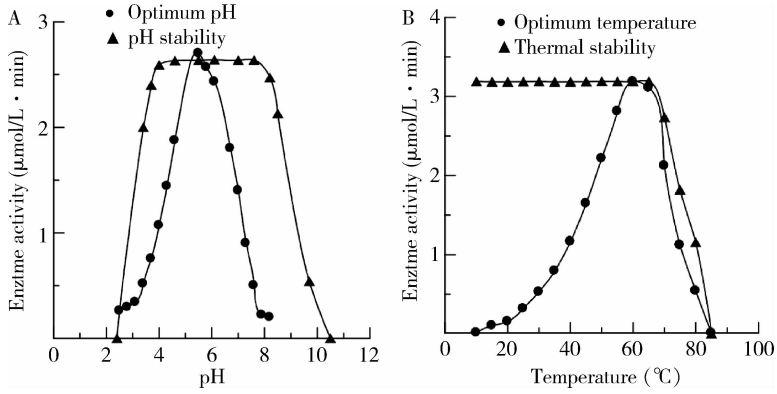

Fig. 5　Effects of pH (A) and temperature (B) on NAGase activity

3.4　The activation energy of NAGase

Activation energy (E_a), the energy which is required during the reaction changing molecules from initial state to transition state, can reflect the degree of enzymatic reaction. Activation energy of NAGase was assayed by measuring the maximum velocity of NAGase for the hydrolysis of pNP-NAG. The plot of $\lg V_m$ versus $1/T$ was shown in Fig. 6. The activation energy was calculated to be 64.8 kJ/mol.

3.5　Effects of metal ions on NAGase activity

The effects of some metal ions on the enzyme activity were surveyed and the results showed that the heavy metal ions Al^{3+}, Pb^{2+}, Cu^{2+} and Zn^{2+} significantly inhibited the NAGase activity (Fig. 7). On the other hand, other metal ions like Na^+, K^+, Mg^{2+}, Ca^{2+} and Ba^{2+} we detected and EDTA had no significant effects on enzyme activity (data not shown). The effects of metal ions Pb^{2+}, Zn^{2+}, Cu^{2+} and Al^{3+} on the NAGase activity showed that the inhibition rates of the four metal ions was $Al^{3+} > Cu^{2+} > Pb^{2+} > Zn^{2+}$.

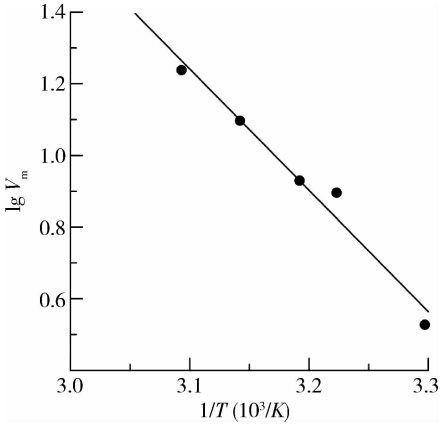

Fig. 6　Activation energy of NAGase for the hydrolysis of pNP-NAG

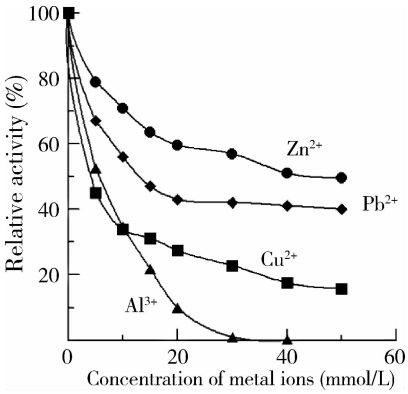

Fig. 7 Effects of metal ions on NAGase activity
The relative activity percentage was the ratio of the specific activity of NAGase treated with metal ions to that of untreated NAGase.

4 DISCUSSION

Little is known regarding NAGase function in honey bees, which has been better studied in some dipteran species. Most cells possess glycosidases as soluble lysosomal proteins that are involved in hydrolysis of complex carbohydrates. In this study, we purified and characterized NAGase from *A. m. ligustica* larvae. The obtained results showed that purified NAGase was achieved by means of $(NH_4)_2SO_4$ fractionation (40% – 70% of saturation), DEAE-cellulose (DEAE-32) ion-exchange chromatography and Sephadex G-100 gel filtration.

NAGase from honey bee larvae could combine with DEAE-32 anion-exchange column at pH 7.5, indicating that its pI was below 7.5. Our deduction was proved to be correct by the result of IEF-PAGE which showed that the pI of NAGase was 4.8. The enzyme was identified to consist of two subunits, which was in accordance with the previous results (Riekenberg *et al.*, 2004; Shi *et al.*, 2007; Liu *et al.*, 2009; Sarosiek *et al.*, 2014). The optimum pH of catalytic reaction of this enzyme was pH 5.5. On the other hand, 90% of enzyme activity was exhibited at pH 4 – 8. The enzyme would be fairly unstable out of this pH range. Besides, the enzyme was rapidly inactivated after incubation for 30 min above 65℃. NAGase from honey bee larvae displayed wider temperature stability as reported in the previous studies (Prody *et al.*, 1985; Xie and Chen, 2004; Shi *et al.*, 2007; Huang *et al.*, 2012; Zhang *et al.*, 2014), possibly because honey bees have wider living area than the other species in the above studies.

The effects of metal ions on enzyme activity showed that NAGase was inhibited by the following metal ions in the decreasing order as: $Al^{3+} > Cu^{2+} > Pb^{2+} > Zn^{2+}$. So we suggest that Al^{3+} was not suitable to be used in pesticides for the consideration of honey bee protection. If worker bees collect the nectar which was sprayed with the pesticide containing Al^{3+}, it would inhibit NAGase activity and might further influence the development of bees.

We used pNP-NAG to assay and validate the characterization of NAGase from honey bee larvae. These new findings will facilitate to further unveil the mechanisms of action of NAGase in the development of honey bees.

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意大利蜜蜂 N-乙酰-β-D-氨基葡萄糖苷酶的分离纯化及性质分析

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摘要：【目标】N-乙酰-β-D-氨基葡萄糖糖苷酶（NAGase）是一种重要的几丁质分解酶，能从 N-乙酰葡萄糖苷的非还原端催化去除 β-1，4-N-乙酰-D-氨基葡萄糖残基，参与了昆虫外骨骼的蜕皮过程。研究蜜蜂该酶的特征有助于阐明其在蜜蜂发育过程中的作用机制。【方法】采用 40% – 70% 硫酸铵分级沉淀、DEAE-纤维素离子交换层析和葡聚糖 G-100 凝胶过滤层析的方法从意大利蜜蜂 *Apis mellifera ligustica* 幼虫体内分离纯化 NAGase。以对-硝基苯-N-乙酰-β-D-氨基葡萄糖苷（pNP-NAG）为底物检测该酶的活力，用 native PAGE 和 SDS-PAGE 检测酶的纯度。IEF-PAGE 测定该酶等电点。葡聚糖 G-200 凝胶过滤层析测定酶的总分子量。【结果】结果显示，纯化的 NAGase 酶的比活力为 803.09 U/mg，总分子量为 77.3 kD。结合 SDS-PAGE 表明该酶由两个具有相同分子量（39 kD）的亚基组成。该酶等电点为 4.8。酶水解底物 pNP-NAG 的过程遵循米氏方程，米氏常数（K_m）和最大反应速度（V_m）分别为 0.11 mmol/L 和 17.65 μmol/L · min。该酶水解反应的最适 pH 和最适温度分别为 pH 5.5 和 60℃。酶催化 pNP-NAG 反应的活化能为 64.8 kJ/mol。Pb^{2+}，Cu^{2+}，Zn^{2+} 和 Al^{3+} 对该酶有不同程度的抑制作用。【结论】本研究描述了意大利蜜蜂 NAGase 的分离纯化方法及其理化性质，为进一步进行蜜蜂 NAGase 的结构解析和功能研究奠定基础。

关键词：意大利蜜蜂；N-乙酰-β-D-氨基葡萄糖苷酶；纯化；性质分析；动力学

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